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ORIGINAL ARTICLE

High-molecular tenascin-C as an indicator of atypical cells in oral brush biopsies

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Abstract Tumour-invasion like wound healing is characterised by the formation of an extracellular matrix with a high tenascin-C content. The tenascin-C molecule undergoes alternative splicing. Analysis using antibody BC2 indicates that especially the high-molecular tenascin-C (hm tn-C) variants are typically tumour-associated, while distribution in normal tissue is restrictive. This study investigated whether hm tn-C is a suitable indicator of atypical cells with invasive potential in oral brush biopsies. One hundred fifty nine consecutive oral brush biopsies with histopathological diagnoses were analysed for the identification of atypical cells. A standardised haematoxylin and eosin staining plus standardised immunocytochemistry using the monoclonal anti-hm tn-C antibody was performed. The bound hm tn-C antibodies were detected with the streptavidine/alkaline phosphatase technique in the autostainer. Conventional cytology produced four false-positives when identifying atypical cells in brush biopsies of inflammatory/benign hyperproliferative mucosa (specificity 96%), while 10 in 52 carcinomas and three of eight recurrences were not identified (sensitivity 78%). Ten of these 13 non-identified tumours could be marked when adding the hm tn-C assay (increasing specificity to 99%). Combining the two assays also reduced the false-positive outcomes from four to one (increasing sensitivity to 95%). The positive and negative predictive values were 92 and 88% for conventional cytology vs 98 and 97% for the dual assay. (1) A 95%-sensitivity proves hm tn-C assisted conventional cytology to be a suitable means of identifying atypical cells in oral brush biopsies. (2) The positive (98%) and negative (97%) predictive values obtained approximate hm tn-C assisted conventional cytology to laminin-5 (100/97%).

Keywords Oral brush biopsy · Oral exfoliative cytology · Early recognition · Tenascin-C · Oral squamous cell carcinoma

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Introduction

In the 1990s, the renaissance of oral cytology was set on foot when, in the USA, a brush was introduced for

Table 1 Classification and numbers of oral mucosal lesions

Diagnosis	Numbers
Normal oral mucosa, non-smoker	**15**
Inflammatory lesion	**28**
Hyperproliferative lesion	**56**
Oral squamous cell carcinoma	**52**
Carcinoma in situ Tis	2
Oral squamous cell carcinoma T1 (mic)	3(3×G2)
Oral squamous cell carcinoma T1	17(17×G2)
Oral squamous cell carcinoma T2	12(9×G2, 3×G3)
Oral squamous cell carcinoma T3	5(3×G2, 2×G3)
Oral squamous cell carcinoma T4	13(9×G2, 4×G3)
Recidivation of an oral squamous cell carcinoma	**8**
Recidivation of an oral squamous cell carcinoma T1	1(G2)
Recidivation of an oral squamous cell carcinoma T2	2(2×G2)
Recidivation of an oral squamous cell carcinoma T3	1(G2)
Recidivation of an oral squamous cell carcinoma T4	4(3×G2, 1×G3)

T tumour size, *G* histological grading, *mic* micro-invasive

sampling and computer-assisted imaging was first employed as an analytical tool [24]. Unlike exfoliative cytology, the purpose of brush biopsy is to provide access to the deeper layers of oral tissue. It is designed to support the morphological clinical grading of mucosal changes and to facilitate the examining physician's decision on the necessity of scalpel biopsy [21, 23]. Various procedures assisting analysis were developed to increase the sensitivity and/or specificity of the identification of oral squamous cell carcinomas and their precursor lesions [4, 14, 22]. At present, these procedures include assisted imaging analysis (Oral CDx®), DNA cytometry [21] and immunocytochemistry using the γ2 chain of laminin-5 [7, 8].

An invasion by oral squamous cell carcinomas represents a process that is coordinated and spatially structured, with the invasive front showing a breakdown in physiological extracellular matrix structures on the one hand and resynthetisation of a provisional extracellular matrix that explicitly incorporates laminin-5 and high-molecular tenascin-C on the other [2, 10]. After a maximising effect in terms of sensitivity and specificity was established for the γ2 chain of laminin-5 used to immunohistochemically enhance oral transepithelial brush biopsy [7, 8], it is now assumed that cells with an invasive potential can also be marked by tenascin-C, which is found to be overexpressed during malignant transformation [18]. In this context, low and high molecular tenascin-C splicing variants need to be differentiated. While low-molecular tenascin-C will not only serve as an immunohistochemical label of atypical cells in tissue slices but will also identify pre-existing structures such as the basilemma of the oral epithelium or small salivary glands, its high-molecular form solely marks the invasive zone [11].

This prospective cytopathological clinical study was designed to assess the value of conventional brush biopsy in the identification of oral squamous cell carcinomas and their precursor lesions in the first step. The second step was to verify whether immunocytochemical imaging of high-molecular tenascin-C, like the γ2 chain of laminin-5, lent itself to the identification of pre-invasive or invasive squamous epithelial cells in oral brush biopsies, and would thus represent another contribution to rapid and reliable interpretations of brush biopsies from oral lesions.

Patients and methods

The value-based identification of atypical epithelia was analysed in 159 consecutive brush biopsies with a histopathological diagnosis for correlative analysis of the brush biopsy diagnoses: 15 brush biopsies of normal oral mucosa, 28 inflammatory lesions, 56 hyperproliferative lesions, 52 primary neoplastic lesions and 8 recurrent squamous cell carcinomas (Table 1).

Lesions were scraped with a brush (Cytobrush® Plus GT non-sterile, Medscand Medical AB, Malmö, Sweden) applying pressure and rotation. The cells harvested were

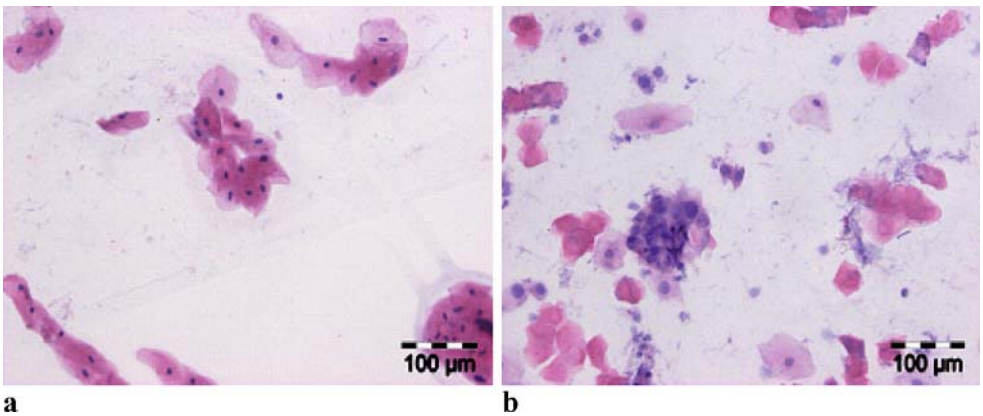

Fig. 1 Standardised HE staining. **a** Normal oral mucosa, large uniform epithelial cells with small uniform nuclei; **b** oral squamous cell carcinoma, highly pathological nucleus-plasma relation, anisomorphy and hyperchromasy of nuclei, cell detritus and bacteria in the background

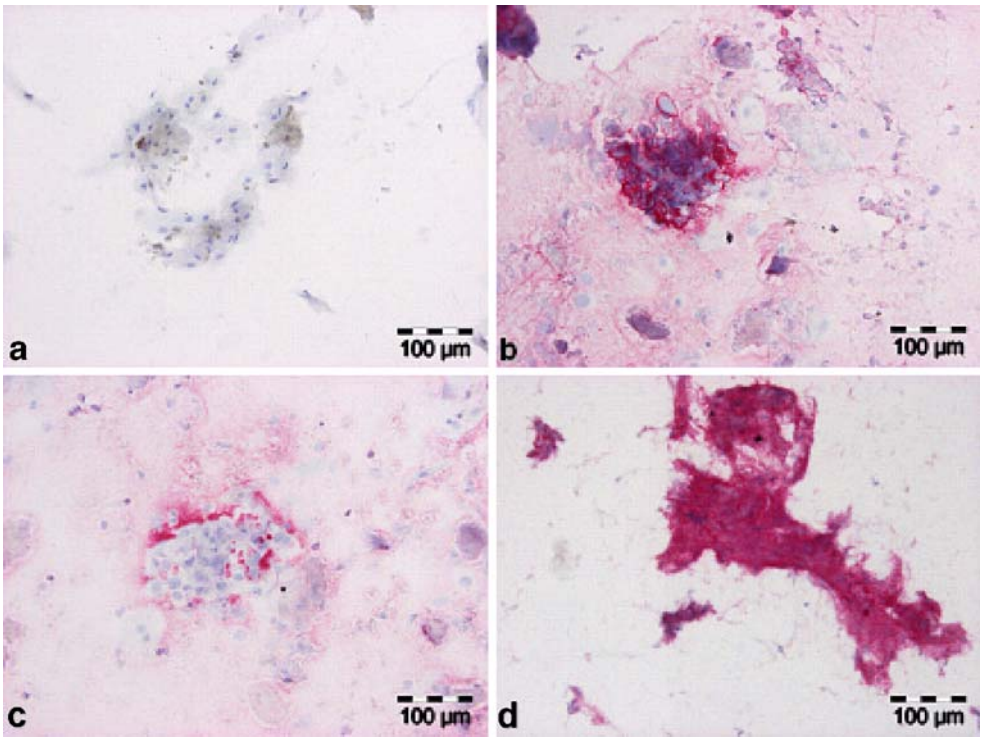

Fig. 2 Standardised immunocytochemistry with high molecular tenascin-C (clone BC2). **a** Normal oral mucosa without immunostaining for hm tn-C, **b** oral squamous cell carcinoma with intensive immunostaining (immunolabelling) of the cytoplasm and of the cell borders of the atypical epithelial complexes, **c** oral squamous cell carcinoma with positive hazes surrounding atypical cells, **d** false positive immunolabelling of bacteria and fungal colonies

then transferred to the slide (SuperFrost® Plus, Menzel, Braunschweig, Germany) by performing full-circle turning and rolling motions with the brush. In this procedure, the brush was repeatedly brought into contact and turned so as to repeat cell patterns on the slide.

For analysis of the brush cytology, standardised haematoxylin and eosin (HE) staining and standardised immunocytochemistry using the monoclonal antibody BC2 (Prof. L. Zardi, National Institute of Cancer Research, Genoa, Italy) against hm tn-C in a dilution of 1:20 were performed

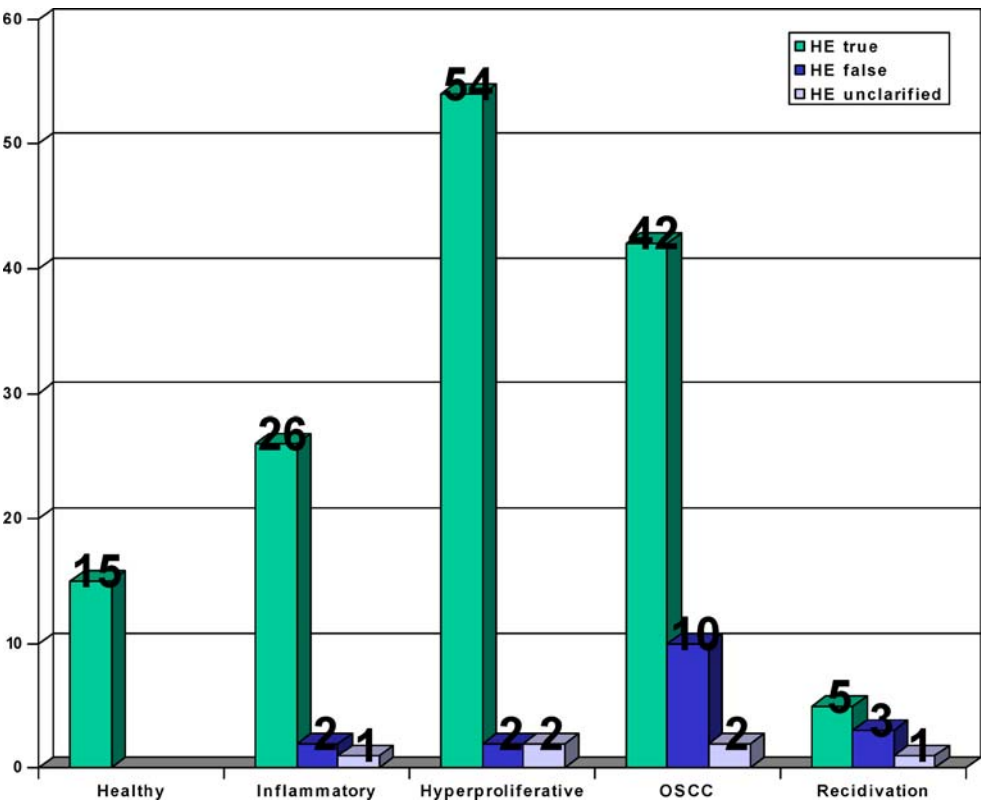

Fig. 3 Result of conventional cytological analysis of brush biopsies. *OSCC* oral squamous cell carcinoma

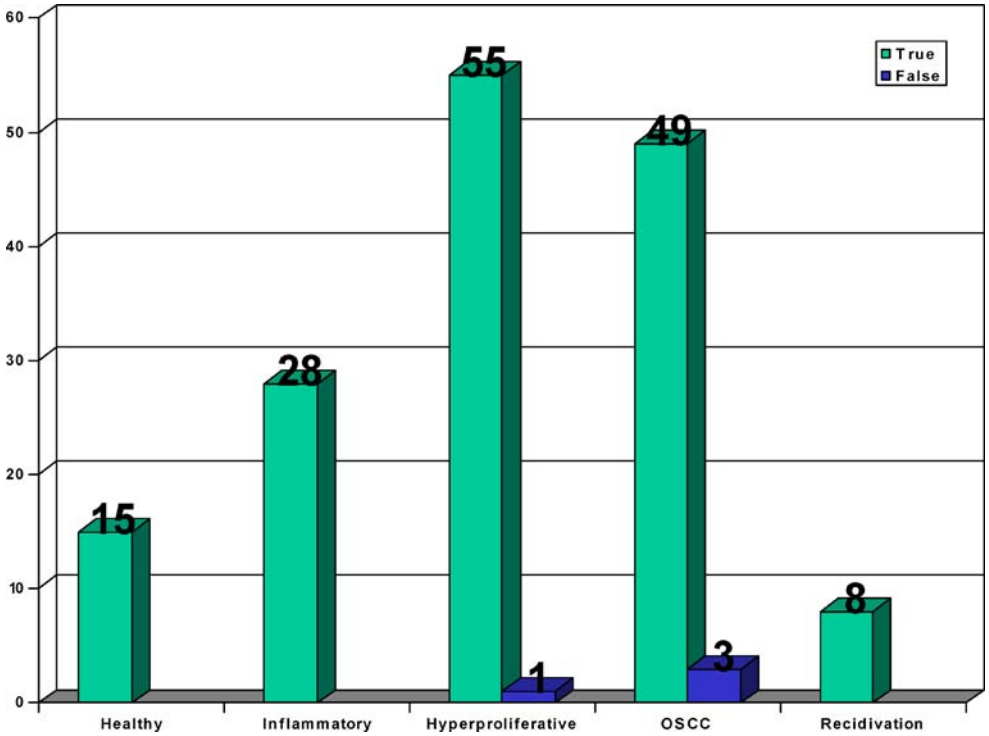

Fig. 4 Result of combination of conventional cytological and immunocytochemical analysis of the brush biopsies. *OSCC* oral squamous cell carcinoma

after acetone fixation. Detection of the bound hm tn-C antibody was performed with the streptavidine/alkaline phosphatase ChemMate kit and the Autostainer (Dako, Glostrup, Denmark) according to the manufacture's protocol. Non-hm tn-C stained cells could be identified in all specimens. Therefore, false-positive immunostains due to technical artefacts could be excluded.

Results

With this brush biopsy procedure, all slides prepared contained cellular material that was qualitatively and quantitatively representative for cytological analysis. The HE stains of the brush biopsies of normal oral mucosa showed large epithelial cells with small uniform nuclei. By contrast, brush cytology of oral squamous cell carcinomas traced many tumour cells that were smaller. They had larger dark blue nuclei and often were arranged in clusters (Fig. 1).

Immunocytochemical evaluation of brush biopsy samples from the normal oral mucosa did not reveal any atypical cells. While no immune staining of high-molecular tenascin-C was demonstrable, the atypical epithelial complexes present in oral squamous cell carcinoma were noticeable by their intense red colouring, which showed one of two immunocytochemical patterns: (1) staining of cytoplasm and cell margins or (2) a positive haze surrounding atypical cells. Bacterial and fungal colonies gave false-positive results (Fig. 2). Moreover, cytology yielded reasonable indications for the specification of reactive lesions.

In six cases, HE screening did not allow for a clear cytological diagnosis. Conventional cytology produced four false-positive cases, i.e. atypical cells in brush biopsies of inflammatory or benign hyperproliferative mucosa. Specificity was 96%. In 13 other cases, a false-negative diagnosis was established: 10 in 52 carcinomas and three of eight recurrences could not be cytologically identified. This resulted in a limited sensitivity of 78% for conventional cytology (Fig. 3).

Ten of these 13 cytologically non-identified tumours could be marked when adding the hm tn-C assay. Enhancing conventional cytology by high-molecular tenascin-C immunocytochemistry also reduced the false-positive outcomes from four to one (Fig. 4).

Combining conventional cytology and immunocytochemistry using high-molecular tenascin-C increased assay specificity from 96 to 99% and sensitivity from 78 to 95%. A positive predictive value of 92% was estimated for conventional cytology and was 98% for the combined immunocytochemistry with high-molecular tenascin-C. The negative predictive value was 88% for conventional cytology and reached 97% for the enhanced assay with high-molecular tenascin-C (Table 2).

The dual assay combining conventional cytology and hm tn-C immunochemistry left a total number of three squamous cell carcinomas unidentified; these included one carcinoma in situ and a micro-invasive carcinoma with a maximum diameter of 0.2 cm.

Table 2 Value-based comparison of conventional and immunocytochemical analysis of brush biopsies with high molecular tenascin-C (hm tn-C)

	Conventional cytology	Tenascin-C immunocytochemistry	Conventional cytology and tenascin-C immunocytochemistry
Specificity (%)	96	93	99
Sensitivity (%)	78	83	95
Positive predictive value (%)	92	88	98
Negative predictive value (%)	88	90	97

Discussion

The limited sensitivity (78%) and specificity (96%) of conventional brush biopsies make it imperative that their sensitivity and specificity be increased with the help of cost-effective and universally available procedures. To date, computer-assisted imaging analysis (Oral CDx®) [22–24, 26], DNA imaging cytometry [13, 21], argyrophilic nucleolar organiser region (AgNOR) analysis [20] and immunocytochemistry using the γ2 chain of laminin-5 [7, 8] have been employed as supporting procedures for the evaluation of oral brush biopsies. The current procedures applied for diagnostic support in conventional brush cytology augment the value and conclusiveness of the

Table 3 Published studies of the value of methodically supported oral brush biopsy as a tool for detecting oral squamous cell carcinoma and its precursor lesions

Author	Method	Patients	Lesions	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Sciubba et al. (1999) [24]	Computer-assisted image analysis (Oral CDx®)	327	327	100	93	90	100
Remmerbach et al. (2001) [19]	Conventional cytology	181	251	94.6	99.5	98.1	98.5
	DNA image cytometry	181	251	96.4	100	100	99
	Conventional cytology combined with DNA image cytometry	181	251	98.2	100	100	99.5
Svirsky et al. (2002) [26]	Computer-assisted image analysis (Oral CDx®)	NN	NN	95.9	NN	38	NN
Remmerbach et al. (2003) [20]	Conventional cytology	75	75	92.5	100	100	84.6
	AgNOR analysis	75	75	100	100	100	100
	Conventional cytology combined with AgNOR analysis	75	75	100	100	100	100
Rick (2003) [22]	Computer-assisted image analysis (Oral CDx®)	NN	NN	90.9	NN	NN	NN
Maraki et al. (2004) [13]	Conventional cytology combined with DNA image cytometry	98	98	100	97.4	89	100
Poate et al. (2004) [16]	Computer-assisted image analysis (Oral CDx®)	49	49	71.4	32	44.1	60
Remmerbach et al. (2004) [21]	Conventional cytology	205	322	91.3	95.1	95.4	92.3
	DNA image cytometry	205	322	95.5	100	100	96.2
	Conventional cytology combined with DNA image cytometry	205	322	97.8	100	100	98.1
Scheifele et al. (2004) [23]	Computer-assisted image analysis (Oral CDx®)	80	96	92.3	94.3	85.7	97.1
Driemel et al. (2006) [8]	Conventional cytology	93	93	79	100	100	92
	γ2-chain of ln-5 immunocytochemistry	93	93	93	98	97	97
	Conventional cytology combined with γ2-chain of ln-5 immunocytochemistry	93	93	93	100	100	97
Present study (2006)	Conventional cytology	159	159	78	96	92	88
	hm tn-C immunocytochemistry	159	159	83	93	88	90
	Conventional cytology combined with hm tn-C immunocytochemistry	159	159	95	99	98	97

NN not named

method (Table 3). Computer-assisted imaging analysis (Oral CDx®), DNA imaging cytometry and AgNOR analysis have limited availability and, due to their technical complexity, are not considered routine procedures. By contrast, immunocytochemistry is a widely used and well-established method in pathological institutions.

Formation of extracellular matrixes with a high tenascin-C content is a characteristic feature of a variety of carcinoma types (endometrial carcinoma [29], colorectal carcinoma [9], gastric carcinoma [31], osteosarcoma [27], breast cancer [1] and oral squamous cell carcinoma [15]). The tenascin-C matrix is synthesised by the carcinoma cells themselves, especially by those of the invasive zone [11, 12, 30], and is already demonstrable in precursor lesions [5, 17, 18, 28]. Excessive tenascin-C of the extracellular matrix is also seen in inflammatory mucosal lesions; matrixes with a high tenascin-C content thus are not an exclusive phenomenon of carcinomas [28]. The tenascin-C molecule undergoes alternative splicing [6]. Unlike "regular" tenascin-C, its high-molecular variants, which can be detected by the antibody BC2, are characteristic of wound healing and tumour tissue [3]. Brush biopsy does not actually identify the extracellular matrix but, rather, its components, which are even demonstrable in epithelial cells, i.e. on an intracellular level. In oral carcinoma cells, the on-going synthesis of high-molecular tenascin-C obviously is so high that it shows distinctively against repair lesions in immunocytological analysis, which is reflected in the fact that high-molecular tenascin-C merely produced four false-positives in 84 inflammatory or benign hyperproliferative lesions. Including high-molecular tenascin-C, analysis can increase the sensitivity and specificity of conventional biopsy from 78 to 96% and from 95 to 99%, respectively. Furthermore, the tenascin-C pattern provides information that is independent of morphology and aids diagnostic interpretation. Immunocytochemical labelling of suspect cells from brush biopsies facilitates the detection of atypical cells in the biopsy preparation and rationalises diagnostics [25].

Oral squamous cell carcinomas were identified by immunocytochemically assisted brush biopsy, irrespective of their histopathological grading (Table 1). With positive and negative predictive values of 98 and 97%, high-molecular tenascin-C comes close to those values achieved by the γ2 chain of laminin-5 [7, 8]. The high sensitivity provided by brush cytology that is immunocytochemically enhanced by high-molecular tenascin-C and the γ2 chain of laminin-5 [7, 8] recommends this technique as a first diagnostic step in the monitoring of mucosal lesions. Positive findings or a progression of lesions, when initial findings were negative, constitute indications for referring a patient to qualified clinical institutions where scalpel biopsy and subsequent histopathological evaluation will be performed.

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